First Observation by Mass Spectrometry of a 3+ Oxidation State for a [4Fe-4S] Metalloprotein: An ESI-FTICR Mass Spectrometry Study of the High Potential Iron-Sulfur Protein from *Chromatium Vinosum*

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Electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FTICR) is used to measure the molecular weight of the high potential iron-sulfur protein (HiPIP) from *Chromatium vinosum* (*C. vinosum*) and its corresponding apoprotein. By accurate mass measurement of the metalloprotein, the oxidation state of the [4Fe-4S] metal center is assigned as 3+. This is the highest oxidation state yet observed by mass spectrometry for a [4Fe-4S] cluster, which usually appears in the 2+ oxidation state. In order to make this assignment correctly, the mass spectrum of the apoprotein was acquired, and a 1 Da difference was found between the molecular mass of the apoprotein and its published amino acid sequence. The mass spectra of the trypsin and cyanogen bromide digests of the alkylated apoprotein were obtained, and the data suggests that the C-terminal glycine residue is amidated. (J Am Soc Mass Spectrom 2001, 12, 819–825) © 2001 American Society for Mass Spectrometry

Iron-sulfur clusters constitute the active site of a number of soluble and membrane-bound proteins [1]. Mass spectrometry has been used to characterize several iron-sulfur proteins, and provides an accurate method to determine the stoichiometry of the inorganic portion of these and other metalloproteins [2–5]. Mass spectrometry also provides a means to measure the oxidation state of metal centers in proteins [2, 6–13]. We have recently examined several iron-sulfur proteins with stoichiometries of Fe_nS_m (n = 1−8, m = 2−8) by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry [2, 7]. Interestingly, the highest biologically relevant oxidation state is the one observed in most of the mass spectra of metalloproteins that have been published. For most [4Fe-4S] proteins, the highest oxidation state reported under biological conditions is 2+, and this is the one that has been observed by mass spectrometry, even though oxidation states ranging from 0 to 4+ are possible [2]. Under reducing, anaerobic conditions, we have recently been able to observe the lowest biologically relevant oxidation state for a number of metalloproteins [14]. Such observations support the contention that the gas-phase properties of protein ions closely resemble their known solution properties. To test this assertion, we have chosen to examine the high potential iron-sulfur protein (HiPIP) from *Chromatium vinosum* [15–24]. This [4Fe-4S] protein can achieve an oxidation state as high as 3+ under biological conditions [15]. The higher oxidation state of this protein results from an interaction of the iron-sulfur cluster with the surrounding amino acids, i.e. the oxidation state is sensitive to the three dimensional structure of the protein, particularly in the region surrounding the metal center. Thus the observation of a 3+ oxidation state for [4Fe-4S] in the mass spectrum of this protein would provide significant evidence that the structure of the protein ion closely resembles that of the solvated molecule.

In order to assign the oxidation state of a metal center in a metalloprotein, the mass of the holoprotein must be measured with an accuracy of at least 1 Da, which is easily achieved with isotopically resolved data such as can be obtained by FTICR mass spectrometry [2, 6, 8, 25]. The difference in mass between the holoprotein and the apoprotein can be assigned to the inorganic portion of the metalloprotein from which the stoichiometry of the metal center can be derived. The oxidation state of the metal center is determined from the difference between the charge present on an ion and the number of excess protons that are present, i.e. the charge of a metalloprotein ion is determined by the number of excess protons and the oxidation state of the metal center. The number of excess protons present on

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a protein ion can be determined by comparing the accurate mass measurement of the ion with the mass calculated for the known elemental composition of the ion. However, post-translational modifications of the protein sequence can interfere with this calculation. For example, amidation of carboxyl groups or deamidation of an amide changes the molecular weight of a protein by 1 Da. As will be illustrated by the data presented below, the potential for errors introduced by such modifications requires that both the apoprotein and holoprotein are accurately measured in order to properly assign the oxidation state of a metal center.

In this paper, we report the analysis of high potential iron-sulfur protein (HiPIP) from *Chromatium vinosum* by ESI-FTICR mass spectrometry, and the identification of a post-translational modification of its C-terminal glycine residue. The original amino acid sequence for this HiPIP was reported in 1973 [26]. The protein was found to contain a single 85 amino acid peptide chain with four cysteine residues that coordinate the metal. The molecular weight of the holoprotein was found to be 9.2 kDa, and the N-terminal and C-terminal ends of the protein were found to be readily accessible to exopeptidases for degradation. The secondary structure of the protein was found by crystallography to contain an antiparallel β-sheet near the C-terminal end of the protein [27]. The N-terminal portion of the protein contains two short helical segments. The protein contains five lysine residues, two arginine residues, and one methionine residue, resulting in eight peptides by tryptic digestion and two peptides by cyanogen bromide cleavage. The tertiary solution structure of the protein has recently been solved for the oxidized form of the HiPIP from *C. vinosum* by NMR [17]. Electrospray ionization mass spectrometry has been used to observe HiPIP holoprotein in both negative and positive ionization modes [28]. HiPIP from *C. vinosum* binds four iron atoms that are coordinated to the protein through cysteine residues 43, 46, 63 and 77. The iron atoms are bridged by inorganic sulfide atoms to form the [4Fe-4S] cluster. The [4Fe-4S] clusters from iron-sulfur proteins can cycle between oxidation states of 1+ to 3+, but no single iron-sulfur center is known to cycle between all three states. The common forms of the clusters cycle between 1+ and 2+, while 2+ and 3+ are the active redox states for the high potential iron-sulfur cluster proteins [15]. HiPIP is of interest to us because it provides an opportunity to observe a higher oxidation for the [4Fe-4S] cluster, and to provide further evidence that mass spectrometry measures a biologically relevant value for the oxidation state of the metal. Since oxidation states of metals exhibit themselves as integer changes in mass, it is important that the mass of the apoprotein, and any post-translational modifications be fully characterized.

Experimental

All mass spectra were acquired with a Bruker BioApex 7 Tesla FTICR mass spectrometer equipped with an electrospray ionization source by Analytica. The glass capillary in the Analytica source was replaced by a heated metal capillary. All samples were ionized by nanoelectrospray. Capillary tips for nanospray were produced in our lab from 100 μm inner diameter fused silica capillary (Polymicro Technologies, Phoenix, AZ). The capillary was pulled to a fine tip by pulling with a constant force while heating with a micro-torch (Microflame, Inc., Minnetonka, MN). The average flow rate for sample introduction to the mass spectrometry was 8 μL/hour. The heated metal capillary was maintained at a temperature between 100–140 °C for the experiments. Ions were stored in the source region in a hexapole ion guide for 0.5–0.75 s and were pulsed through a series of electrostatic lenses into the detection cell where trapping potentials of 0.90 V and 0.95 V were used on the front and back trapping plates of the cell to isolate ions for excitation and detection. The time domain signal (transient) was collected by averaging approximately 50 scans. The summed transient was apodized prior to application of the Fourier transform, followed by calibration to yield a mass spectrum.

The high potential iron-sulfur protein was isolated from *Chromatium vinosum,* and was purified using the procedure outlined by Knaff and coworkers [19]. Apoprotein for the HiPIP was generated by heating the holoprotein at 90 °C for 15 minutes. Disulfide bonds in the denatured HiPIP were reduced before alkylation by incubating the apoprotein (15 μM) with dithiothreitol (4.5 mM) at 45 °C for 15 min [26]. The reduced protein sample was alkylated with iodoacetamide at a concentration of 10 mM. The sample was mixed with a vortex shaker for 30 s and then allowed to rest at room temperature for 5 min. Excess reducing agent was removed by concentrating the sample in a microcentrifuge tube with a 5 kDa cutoff integral dialysis membrane (Millipore Corporation, Bedford, MA) and washed with 200 μL of 15 mM ammonium acetate solution. The alkylated protein was resuspended in water, yielding a final concentration of 15 μM.

Trypsin digestion was performed on both the denatured HiPIP and the alkylated apoprotein. The protein solutions were desalted by buffer exchange with 15 mM ammonium acetate using a microcentrifuge tube with a 5 kDa cutoff integral dialysis membrane. A solution of modified trypsin (Promega, Madison, WI) at a concentration of 2 μg/100 μL in 100 mM ammonium bicarbonate solution was mixed with an equal volume of the 15 μM protein solutions and incubated for 24 hours at 45 °C.

For chemical digestion of HiPIP, cyanogen bromide was dissolved in a solution of 70% formic acid to yield a concentration of 5 mg/mL [29]. 15 μL of the resulting cyanogen bromide solution was added to the lyophilized protein to yield a final concentration of 1 mg/mL

of protein. The digest solution was stirred with a vortex mixer for 30 s and then wrapped in aluminum foil to protect the sample from visible radiation. The sample was allowed to react for 22 h. The solution was then lyophilized and the resulting peptides were resuspended in a solution of 49% methanol, 49% water and 2% acetic acid.

The FTICR data produces isotopically-resolved mass spectra from which the monoisotopic molecular weight of the protein is determined. Monoisotopic molecular weight is defined as the sum of the lowest molecular weight isotopes in the molecular formula, and for most proteins, this corresponds to ^{12}C, ^{1}H, ^{14}N, ^{16}O, and ^{32}S. A comparison of theoretically derived isotope distribution with experimental data identifies the monoisotopic peak in the experimental data, from which the molecular weight may be calculated [30]. For metalloproteins, the definition of monoisotopic peak is slightly different than for other proteins. The monoisotopic peak for a metalloprotein is defined here as the sum of all of the lowest molecular weight isotopes in the molecular formula of the protein plus the mass of the most abundant isotope of the metal or metals that make up the metalloprotein. This corresponds to ^{56}Fe instead of ^{54}Fe, since the natural abundance of ^{54}Fe is 5.82%, and produces a peak that is usually too small to be identified. Molecular weights for most proteins are calculated assuming all of the ionizable sites in the protein are neutral, but metalloproteins provide an additional challenge for mass spectrometry since the metal or metal cluster can also carry charge [2, 7, 8]. We use apparent mass to refer to the molecular weight that is derived assuming all of the charge present on an ion is due to excess protons. Calculated mass is the mass derived from the sum of all of the lowest molecular weight isotopes in a given molecular formula (with the exception of iron, as described above). This is identical to the monoisotopic mass except when disulfide bonds are present in the molecule of interest. The calculated mass makes no assumptions about the formation of disulfide bonds in a protein. The difference in mass between the calculated mass and the apparent mass allows one to assign the oxidation state of the metal center and the number of disulfide bonds in the molecule. The apparent and calculated masses can differ by as little as one Da in the case of oxidized versus reduced metal or two Da per disulfide bond within the molecule. This procedure has been described in detail in an earlier publication [2].

Results and Discussion

Figure 1 shows the mass spectrum of a typical [4Fe-4S] protein, ferredoxin from *Pyrococcus furiosus* (*Pf*). With the nondenaturing conditions used here, only one major and two minor charge states appear in the mass spectrum. An expansion of the major charge state shows a peak corresponding to the intact holoprotein and a smaller peak corresponding to an ion 16 mass units higher in molecular weight. Such satellite peaks are common with iron-sulfur proteins [2], and is thought to be from to oxidation of one of the cysteinyl sulfurs in the binding region of the metal center. The [4Fe-4S] cluster is coordinated to the protein through three cysteine residues and one aspartic acid residue [31]. The apparent mass calculated for the protein is 7509.83 Da and the calculated mass for the protein is 7513.87 Da. The difference of 4 Da supports the assignment of a 2+ oxidation state for the metal center and a single disulfide bond between two cysteines in the protein (C21 and C48). The oxidation state of the metal was confirmed in previous work in which mutation of one the cysteines to alanine (C21A) eliminated the disulfide bond [2]. The highest oxidation state observed by other methods for *Pf* ferredoxin is 2+ [31]. Several similar examples of iron-sulfur proteins have been recently published that establish that mass spectral data provide a way to determine the highest biologically relevant oxidation state of the metal center of a metalloprotein [2, 7].

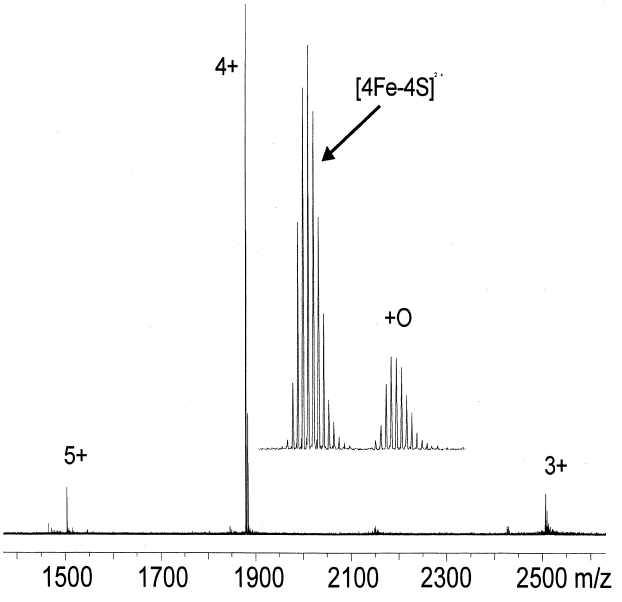

Figure 1. The ESI-FTICR mass spectrum of ferredoxin from *Pyrococcus furiosus* containing a [4Fe-4S] cluster. The measured molecular weight for the protein is consistent with the presence of a single disulfide bond (C21–C48) and an oxidation state for the metal cluster of 2+.

HiPIP from *C. vinosum* is similar to *Pf* ferredoxin in that it contains a [4Fe-4S] metal center. The ESI-FTICR mass spectrum for HiPIP from *C. vinosum* is shown in Figure 2. The 4+, 5+ and 6+ charge states are observed, corresponding to protein with an intact [4Fe-4S] cluster. In addition, the 9+ charge state of HiPIP dimer containing 2 intact [4Fe-4S] clusters is observed in the mass spectrum obtained under nondenaturing conditions (top). The isotope distribution in the mass spectrum was matched against a calculated distribution in order to assign the monoisotopic peak. As can be seen in the

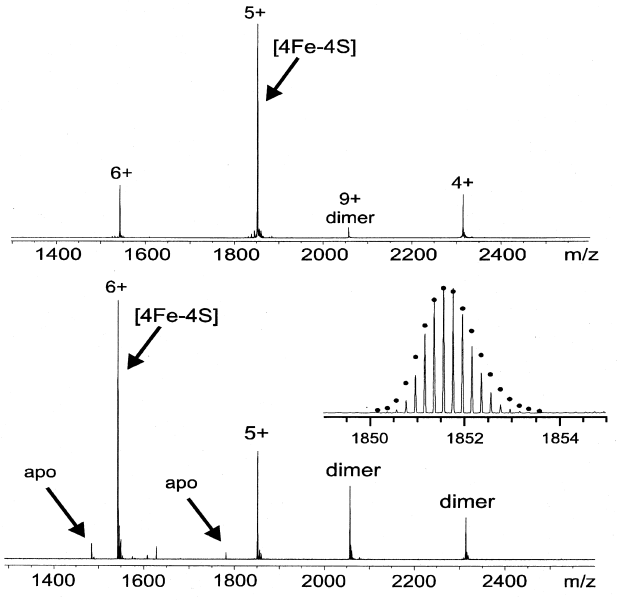

Figure 2. (Top) The ESI-FTICR mass spectrum of high potential iron-sulfur protein (HiPIP) holoprotein from *C. vinosum* under nondenaturing conditions. The small peak at mass-to-charge 2027 arises from HiPIP dimer. (Bottom) The ESI-FTICR mass spectrum of HiPIP to which apoprotein was added as an internal calibrant. The inset compares theoretical (circles) and experimentally-observed isotope abundances for the 5+ charge state.

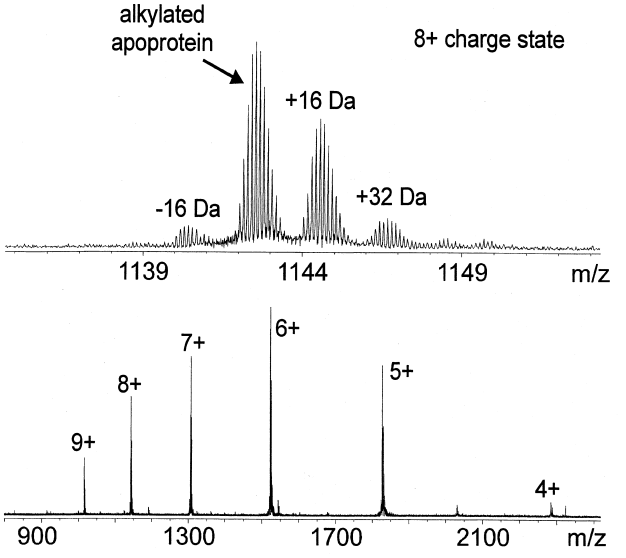

Figure 3. The ESI-FTICR mass spectrum of HiPIP apoprotein from *C. vinosum*, in which the cysteines have been alkylated by iodoacetamide. (Top) An expansion of the 8+ charge state. (Bottom) The entire mass spectrum with a charge state distribution from 4+ to 9+.

figure, the most abundant peaks match the expected distribution, while the lower abundance peaks appear smaller than expected. This is typically observed with this instrument, and allows the confident assignment of the monoisotopic mass. The spectrum was calibrated externally using ubiquitin, and the apparent mass obtained for the holoprotein is 9247.77 Da, while the mass calculated from the reported amino acid sequence is 9251.86 Da. Since the protein only contains 4 cysteine residues and all of them are involved in cluster ligation to the protein, the mass difference cannot be a result of disulfide bond formation. Rather, the difference of 4 mass units suggests a 4+ charge state on the metal cluster. Since the inorganic sulfide ions have a formal oxidation state of 2−, and iron could conceivably be present as Fe^{3+}, an overall charge on the cluster of 4+ may seem reasonable. However, prior solution studies suggest that the highest oxidation state for this cluster is 3+ in this protein [17]. To verify the 4 Da difference between apparent and calculated masses, apoprotein was added as an internal calibrant to the holoprotein solution and a mass spectrum was obtained. The resulting spectrum is shown at the bottom of Figure 2. Apoprotein in the 5+ and 6+ charge states were used for the internal calibration assuming that the free cysteine residues that normally ligate the iron-sulfur cluster were oxidatively coupled after cluster removal. This assumption is consistent with previous measurements of ferredoxin apoprotein and other denatured metalloproteins [2]. The molecular weight of the apoprotein was derived from the published amino acid sequence [26]. After internal calibration with the apoprotein peaks (using the published amino acid sequence for this protein), the apparent mass of the holprotein that was derived from the 6+ charge state was 9248.88 Da, one mass unit higher than by external calibration. From this value, one derives a 3+ oxidation state for the metal cluster, which agrees with literature values for the oxidized form of the cluster [15], and provides further support for our contention that the highest biologically relevant oxidation state is the one produced by nondenaturing electrospray ionization.

The mass difference of 1 Da between the molecular weights determined by internal calibration with the apoprotein versus external calibration with a well characterized peptide standard suggests that the HiPIP structure reported in the literature, which was used to calculate the mass for internal calibration, is incorrect, and that the protein weighs one mass unit less that the molecular weight calculated from the published sequence [26]. Our assignment of 2 disulfide bonds in the HiPIP apoprotein was confirmed by alkylating the apoprotein to prevent the formation of disulfide bonds before detection. The resulting mass spectrum is shown in Figure 3. The apparent mass that is derived from the data obtained from the mass spectrum is 9127.29 Da, 1 amu less than the value calculated from the literature sequence, 9128.32 Da. This difference suggests a difference between the published sequence and the isolated protein such as a post-translational modification of one of the amino acids.

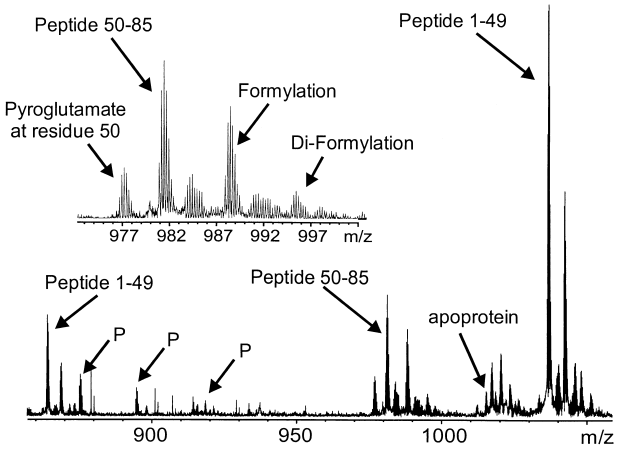

Figure 4. The ESI-FTICR mass spectrum of the cyanogen bromide digest of the alkylated apoprotein of HiPIP. The C-terminal fragment mass is found to be 1 Da less than its predicted mass. A small amount of undigested apoprotein is also observed in the mass spectrum. Peaks labeled "P" result from polyethylene glycol from the containers used for the chemical digestion. (Inset) An expansion of the region of the spectrum showing peptide 50–85 (T_2) and additional adduct peaks.

The location of the modification was first examined by conducting a cyanogen bromide digestion of the alkylated protein, which is expected to cleave on the C-terminal side of the single methionine residue at position 49, near the middle of the protein. The mass spectrum obtained after digestion is shown in Figure 4. The expected and measured masses of the resulting peptides are shown in Table 1. Two charge states of the N-terminal fragment were detected from which an apparent mass of 5177.35 Da was derived, in excellent agreement with the calculated mass for this fragment, 5177.44 Da. The measured mass for the C-terminal fragment is 3919.80 Da, one mass unit less than the value calculated from the published sequence, 3920.86 Da. This confirms that a modification has occurred in one of the amino acids of the C-terminal half of the protein that reduces the mass by 1 Da. Peaks are also observed in the mass spectrum that represent fragments containing homoserine and homoserine lactone as well as formylation of amino groups in the peptides. The masses of these peaks are in agreement with a 1 mass unit shift in the C-terminal fragment. Low intensity peaks from undigested apoprotein were also detected.

Table 2. Tryptic fragment calculated and measured masses for cysteine-alkylated HiPIP

Tryptic Fragment	Peptide Sequence	predicted mass (Da)	measured mass (Da)
T_1	SAPANAVAADDATAIALK	1668.87	1668.85
T_2	YNQDATK	838.38	838.37
T_3	SER	390.19	Not Detected
T_4	VAAAR	486.29	486.28
T_5[a]	PGLPPEEQH(C)AN(C)QF MQADAAGATDEWK	3157.33	3157.24
T_6	G(**C**)QLFPGK	905.44	905.43
T_7	LINVDGW(**C**)ASWTLK	1661.82	1661.81
T_8	AG	146.07	Not Detected

[a] Fragment T_5 is unexpected because of the proline residue at its N-terminus, but is nevertheless observed.

To more precisely locate the modification to the published sequence, we examined a tryptic digest of the protein for which cysteines were alkylated by iodoacetamide. Table 2 compares predicted and measured masses for the tryptic fragments of the alkylated protein. Of the eight fragments that are expected, six were detected in the ESI-FTICR mass spectrum, shown in Figure 5. Undetected fragments were fragment T_3, which consists of amino acids SER, and fragment T_8, which consists of amino acids AG. Since SER resides in the N-terminal half of the protein, the results of the cyanogen bromide digest eliminate T_3 as a modified fragment, and suggests that AG, fragment T_8, is modified. The one mass unit decrease from the expected value suggests amidation, probably by modification of glycine in the AG residue at the C-terminus of the protein.

Since the AG residue was not observed in the trypsin digest of the alkylated protein, further measurements were made to try to directly observe this residue. A trypsin digest was produced for the nonalkylated apoprotein. Some fragments of the protein were found to be linked via disulfide bonds, and peaks due to incomplete digestion of the protein were also observed. Nevertheless, complete coverage of the protein was obtained from the mass spectral data shown in Figure 6. The predicted masses and measured masses are listed in Table 3. A peak corresponding to the fragment T_7+T_8 arises from incomplete digestion at a lysine residue. These data show the preference of trypsin for arginine compared to lysine [19]. The peaks of greatest interest in the mass

Table 1. Peptide fragment masses resulting from cyanogen bromide digestion of HiPIP

CNBr Fragment	Peptide Sequence	predicted mass (Da)	measured mass (Da)
F_1	SAPANAVAADDATAIALKYNQDATK SERVAAARPGLPPEEQHCANCQFM	5177.44	5177.35
F_2	QADAAGATDEWKGCQLFPGKLINV DGWCASWTLKAG	3920.86	3919.80

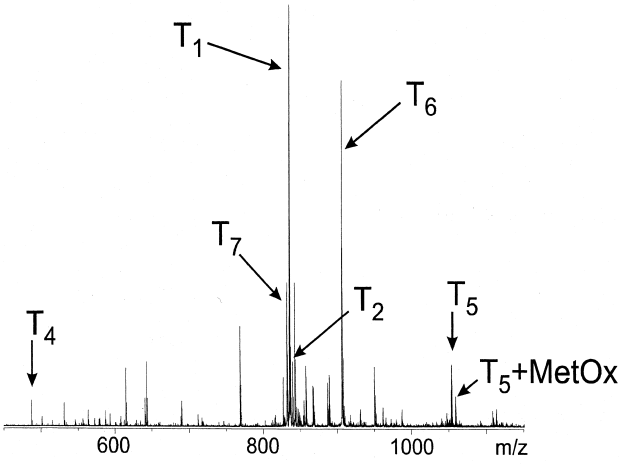

Figure 5. The ESI-FTICR mass spectrum of the tryptic digest of the alkylated HiPIP apoprotein. Of eight possible fragments, six are found in the mass spectrum (see Table 2). From these data, the 1 Da mass difference for the complete protein can be assigned to a modification in either T_3 (SER) or T_8 (AG).

spectrum are labeled as fragment T_6+T_7 and fragment T_7+T_8. The apparent mass for the peak labeled T_6+T_7 is 2451.10 Da and the calculated mass is 2453.22 Da. Although each of the two fragments has been cleaved at both C-terminal and N-terminal residues by trypsin, they are still linked by a disulfide bond which is consistent with the 2 Da mass difference between the apparent and calculated masses. The C-terminal fragment, T_7+T_8, has an apparent mass of 1731.86 Da and a calculated mass of 1732.86 Da. Since residue T_7 agrees with its predicted mass (from the trypsin digest of the alkylated protein), these data confirm that the C-terminal fragment

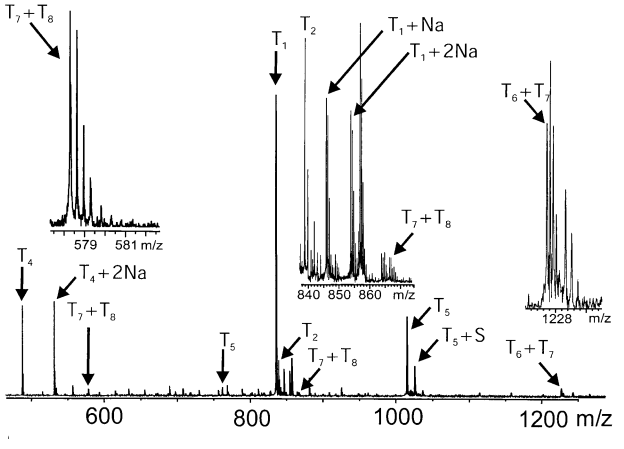

Figure 6. The ESI-FTICR mass spectrum of the tryptic digest of the non-alkylated protein. Complete coverage of the protein is observed in the mass spectrum. The measured mass of $T_6-S-S-T_7$ agrees with its calculated value, while T_7+T_8 has an apparent mass that is 1 Da less than its calculated value, indicating that the modification is in T_8, the dipeptide, AG.

consisting of amino acids alanine and glycine has been modified to produce a decrease in mass of 1 Da. Side chain modification is unlikely, so amidation of the C-terminal glycine is deduced from these data. This is a fairly common modification in eukaryotes, but can also occur in bacteria [32].

Enzymes of high specificity are of great importance in the analytical applications of protein digestion [33]. Rules governing amino acid cleavage are important because of their use in the interrogation of database proteins that generate theoretical fragments for comparison to experimentally derived fragment masses. All digestion methods give some heterogeneity in specificity of bond cleavage, so products must be characterized in detail [34]. The difficulties that arise from this heterogeneity are synonymous with the problems that are found in the detection of post-translational modifications in that these occurrences can not be predicted. An example of such an unpredicted cleavage in these data is peptide T_5, resulting from cleavage of a R–P bond. Although peptide bonds between basic residues and proline (RP or KP) are usually immune from trypsinolysis, cleavage can occur in rare cases [34], and is observed here.

Conclusion

A 3+ oxidation state is assigned to the [4Fe-4S] center of HiPIP from *C. vinosum*. This is the naturally occurring oxidized state of the metal center under biological conditions, and provides yet another example that metalloproteins exhibit the highest biologically relevant oxidation state when analyzed using electrospray ionization mass spectrometry. The highest oxidation state achieved by the [4Fe-4S] cluster depends in a sensitive manner on the environment of the metal center, specifically, the presence of specific amino acids in the coordination sphere of the metals. These data suggest that the three dimensional structure of the protein is retained to a point late in the ionization process, and perhaps even in the gas phase.

The previously unknown amidation of the C-terminal glycine residue of the high potential iron-sulfur protein from *C. vinosum* has been identified through ESI-FTICR mass spectrometry. Such a modification produces only a 1 Da decrease in the molecular weight of the molecule, but this is readily discerned by FTICR mass spectrometry. The proper assignment of the molecular weight of this protein is required to determine the oxidation state of the metal center. The identification of such small mass shifts in an ion that weighs approximately 10 kDa demonstrates the utility of FTICR mass spectrometry for bioanalytical measurements.

Table 3. Peptide fragment masses for tryptic digestion of the non-alkylated HiPIP

Tryptic Fragment	Peptide Sequence	predicted mass (Da)	measured mass (Da)
T_1	SAPANAVAADDATAIALK	1668.87	1668.79
T_2	YNQDATK	838.38	838.35
T_3	SER	390.186	Not Detected
T_4	VAAAR	486.29	486.27
$T_5(S-S)$[a]	PGLPPEEQH**C**AN**C**QFMQA DAAGATDEWK	3041.27	3041.20
T_6	GCQLFPGK	848.42	Not Detected
T_7	LINVDGWCASWTLK	1604.80	Not Detected
T_8	AG	146.07	Not Detected
T_2-T_3			
$T_6-S-S-T_7$[a]	G**C**QLFPGK + LINVDGW**C**ASWTLK	2451.20	2451.10
T_7-T_8	LINVDGWCASWTLK-AG	1732.86	1731.86

[a] Predicted masses take into account the two mass unit difference from disulfide bond formation.

Acknowledgments

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